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In TNF-stimulated Cells, RIPK1 Promotes Cell Survival by Stabilizing TRAF2 and cIAP1, which Limits Induction of Non-canonical NF-κB and Activation of Caspase-8*[S]

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RIPK1 is involved in signaling from TNF and TLR family receptors. After receptor ligation, RIPK1 not only modulates activation of both canonical and NIK-dependent NF-κB, but also regulates caspase-8 activation and cell death. Although overexpression of RIPK1 can cause caspase-8-dependent cell death, when RIPK1$^{-/-}$ cells are exposed to TNF and low doses of cycloheximide, they die more readily than wild-type cells, indicating RIPK1 has pro-survival as well as pro-apoptotic activities (1, 2). To determine how RIPK1 promotes cell survival, we compared wild-type and RIPK1$^{-/-}$ cells treated with TNF. Although TRAF2 levels remained constant in TNF-treated wild-type cells, TNF stimulation of RIPK1$^{-/-}$ cells caused TRAF2 and cIAP1 to be rapidly degraded by the proteasome, which led to an increase in NIK levels. This resulted in processing of p100 NF-κB2 to p52, a decrease in levels of cFLIP$_L$, and activation of caspase-8, culminating in cell death. Therefore, the pro-survival effect of RIPK1 is mediated by stabilization of TRAF2 and cIAP1.

In mouse embryonic fibroblasts (MEFs),[2] TNFR1 signaling is mediated by binding of the adaptor protein TRADD to its cytoplasmic domains. TRADD then recruits TRAF2 and RIPK1. TRAF2 binds to cIAP1 and cIAP2, which mediate ubiquitylation of RIPK1 (3–5). A series of ubiquitylation and phosphorylation events follow, leading to activation of TAK1, p65/RelA NF-κB, and JNK (6–8). When pro-survival signals mediated by NF-κB are blocked, for example by deletion of genes for p65/RelA, or addition of the translation inhibitor cycloheximide, ligation of TNFR1 results in recruitment of FADD, binding and activation of caspase-8, and apoptosis.

Ligation-induced recruitment of RIPK1 to TNFR1 was thought to be necessary for activation of the IKK complex that phosphorylates IkB prior to its ubiquitylation by SCF^{BTrCP}. In this way, RIPK1 was proposed to promote cell survival by allowing activation of the canonical NF-κB. Consistent with this model, gene deletion of RIPK1 sensitizes cells to killing by TNF in combination with low doses of cycloheximide (2). However, we have recently shown that RIPK1 is not essential for activation of canonical p65/RelA NF-κB in response to TNF, but plays a redundant role (1). This suggests that the survival effect of RIPK1 in response to TNF is mediated by some other mechanism.

Like RIPK1$^{-/-}$ cells, those deficient for TRAF2 are also more likely to die than wild-type MEFs when treated with low doses of cycloheximide and TNF. This sensitization was shown to be due to loss of function of the RING domain of TRAF2 (9). The fact that RIPK1 has been shown to interact directly with TRAF2 raised the possibility that they act in concert to inhibit cell death in response to TNF, but to date this has not been confirmed, and the mechanism has not been elucidated (10).

We reasoned that the ability of TRAF2 and RIPK1 to promote survival of TNF-treated cells might be related. To test this hypothesis we compared TRAF2$^{-/-}$ and RIPK1$^{-/-}$ gene-deleted cells. Here we show that RIPK1 prevents activation of non-canonical NF-κB in response to TNF by protecting TRAF2 and cIAP1 from degradation. In both RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs, cFLIP$_L$ is destabilized and rapidly degraded when TNF is added. In contrast, the cleaved form of cFLIP, p43cFLIP, remains stable, and is able to protect cells unless transcription or translation are inhibited.

MATERIALS AND METHODS

Cell Lines—Wild-type and knock-out MEFs were generated from E15 embryos in accordance with standard procedures and were infected with SV40T expressing lentivirus. All cell lines were maintained at 37 °C, 10% CO_2 in DMEM supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine and passaged twice weekly. RIPK$^{+/-}$ mice were a kind gift from Michelle Kelliher and TNFR1$^{-/-}$ mice were a gift from Heinrich Korner. Thymocytes were isolated from timed matings as previously described (1). To generate murine keratinocytes the skin of E19 embryos was incubated overnight in 2.1 units/ml dispase II (Roche) and gentamicin supplemented keratinocyte serum-free media (KSFM; Invitrogen). The epidermis was then separated from the dermis and trypsinized to isolate epidermal keratinocytes which were cultured in KSFM.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: MEF, mouse embryonic fibroblast; PI, propidium iodide; CHX, cycloheximide; DISC, death-inducing signaling complex.

Death Assays—Cells were seeded on 12 well tissue culture plates at ~50% confluency and were allowed to adhere for 16–20 h. Human Fc-TNF (100 ng/ml), or CHX (250 ng/ml) were added to cells for 24 h and cell death measured by PI staining and flow cytometry. In each sample 10,000 events were measured, and the survival (% PI-negative cells) quantified.

Nuclear Fractionation—MEFs were treated with TNF for the indicated times and then cells were harvested and extracts made using the NE-PER® Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) as per manufacturer's instructions. Fractions were analyzed by Western blot.

Western Blotting—Cells were treated with 100 ng/ml of Fc-TNF or recombinant mmTNF for the times indicated. MG132, necrostatin, NH_4Cl, and chloroquine were added 1 h prior to TNF addition. To remove them, cells were treated with trypsin and washed in ice-cold PBS, and lysed in DISC buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, Roche complete protease inhibitor mixture, 5 μM NEM) on ice. Cell lysates were spun at 14,000 × *g* for 10 min and the soluble material retained. For insoluble fractions, the pellet was resuspended in 1% SDS with 10 mM Tris, pH 7.4 and boiled for 10 min. Samples were then sonicated briefly to shear DNA, and then boiled for 5 min with sample loading buffer. Samples were separated on 4–12% polyacrylamide gels (Invitrogen), and transferred to nitrocellulose membranes for antibody detection. All membrane-blocking steps and antibody dilutions were performed with 5% skim milk in PBS containing 0.1% Tween 20 (PBS-T), and washing steps performed with PBS-T. After incubating with HRP-coupled secondary antibodies, Western blots were visualized by ECL (Amersham Biosciences, UK and Millipore).

Reagents and Antibodies—The primary antibodies used were anti-β-actin (A-1978, Sigma), anti-RIPK1 (610458, BD Transduction Laboratories), anti-cFLIP (XA-1008, ProScience), anti-TRAF2 (sc-876, Santa Cruz Biotechnology), anti-cIAP1 (in house), anti-PARP (9542, Cell Signaling Technology), anti-caspase-8 (Gift from Lorraine O'Reilly, Walter and Eliza Hall Institute), anti-NIK (4994, Cell Signaling Technology), anti-NF-κB2 P100 (4882, Cell Signaling Technology), anti-Lamin A/C (20681, Santa Cruz Biotechnology). Proteasome and lysosomal inhibitors MG132 (Saphire Biosciences) and chloroquine (Sigma). RIPK1 kinase inhibitor necrostatin-1 (Sigma), Fc-TNF and FC-FasL (in house), Iso Leu TRAIL (gift from Christoph Emmerich), soluble mmTNF (315001a, peprotech), compound A (TetraLogic Pharmaceuticals).

RESULTS

In Response to TNF, TRAF2, and cIAP1 Remain Stable in Wild-type Cells but Are Degraded in RIPK1$^{-/-}$ MEFs—Like RIPK1$^{-/-}$ MEFs, those derived from TRAF2$^{-/-}$ mice have increased sensitivity to induction of apoptosis by TNF (11). It has been reported that cFLIP levels decline when TRAF2$^{-/-}$ MEFs are treated with TNF, whereas they remain stable in wild-type MEFs (3). To determine whether a decline in cFLIP following TNF treatment might also explain the increased sensitivity of RIPK1$^{-/-}$ MEFs to TNF, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with TNF for 1 h, and Western analysis was performed to determine levels of TRAF2, cIAP1, and cFLIP (Fig. 1*A*). Although resting levels of $cFLIP_L$ were the same in all cell genotypes, when TNF was added $cFLIP_L$ was lost in both RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs, but not wild-type MEFs. In addition to the loss of $cFLIP_L$ in the RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs, there was also a striking decrease in the levels of TRAF2 and cIAP1 in TNF-treated RIPK1$^{-/-}$ MEFs (Fig. 1*A*, *lane 4*). TRAF2 loss was confirmed in multiple cell lines derived from independent embryos (supplemental Fig. S1).

TRAF2 and cIAP1 form a complex with TRAF3 and cooperate in ubiquitylation and degradation of NIK (NF-κB-inducing kinase) (12, 13). When TRAF3 levels were examined under the same treatments, there was no observable degradation (data not shown).

Because treatment with the TNF-related cytokine TWEAK or TNF itself causes re-localization of TRAF2 and cIAP1 (14–17), we wondered whether their re-localization would be affected by deletion of RIPK1. To test this, we first treated RIPK1$^{-/-}$ MEFs with TNF and lysed them in death-inducing signaling complex (DISC) lysis buffer and separated detergent soluble and insoluble fractions. These were then analyzed by Western blot for levels of cIAP1 and TRAF2 (Fig. 1*B*). Unlike normal MEFs, in TNF-treated RIPK1$^{-/-}$ cells the majority of TRAF2 and cIAP1 disappeared from the soluble fraction, and simultaneously increased in the insoluble fraction within 15 min (Fig. 1*B*). The amount of TRAF2 in the insoluble fraction subsequently decreased. Interestingly, in the insoluble fraction TRAF2 appeared to be a doublet, with one form migrating more slowly, and the other more rapidly than TRAF2 in the soluble fractions.

Like TRAF2, cIAP1 was rapidly lost from the soluble fraction in RIPK1$^{-/-}$ cells, but unlike TRAF2, there was no apparent transfer of cIAP1 from the soluble to the insoluble fraction at 15 min. Instead, cIAP1 seemed to be degraded before it could be detected in the insoluble fraction (Fig. 1*B*). The speed of this trafficking and degradation was more rapid than that observed following treatment with TWEAK, which requires hours to cause degradation of TRAF2 and cIAP1 (14, 18).

We next pretreated the MEFs with either the proteasome inhibitor MG132, or the lysosomal inhibitors NH_4Cl and chloroquine, and exposed them to TNF for 1 h. Cells were then lysed in DISC buffer and separated into soluble and insoluble fractions and analyzed by Western blot. The proteasome inhibitor MG132 was able to reduce degradation of TRAF2 and cIAP1 (Fig. 1*C*, *lanes 3* and *8*). Both of the lysosomal inhibitors provided some protection to TRAF2 in the insoluble fraction, but less for cIAP1 (Fig. 1*C*, *lanes 4*, *5*, *9*, *10*). cIAP1 accumulated in the insoluble fraction of MG132-treated cells, indicating that cIAP1 is recruited to the insoluble fraction prior to degradation, as occurs with TRAF2. Therefore in the absence of RIPK1, addition of TNF triggers degradation of both TRAF2 and cIAP1 by the proteasome. Partial blockage of degradation of TRAF2 and cIAP1 by lysomal inhibitors may suggest, at least in part, that degradation occurs in lysosomes too.

The cIAP1 that accumulated in the insoluble fraction with MG132 treatment appeared to be modified in a pattern consistent with ubiquitylation. To confirm the modification of TRAF2 and cIAP1, RIPK1$^{-/-}$ MEFs were treated with TNF for 0, 5, and 15 min and cells lysed in DISC or SDS buffer (Fig. 1*D*). There is

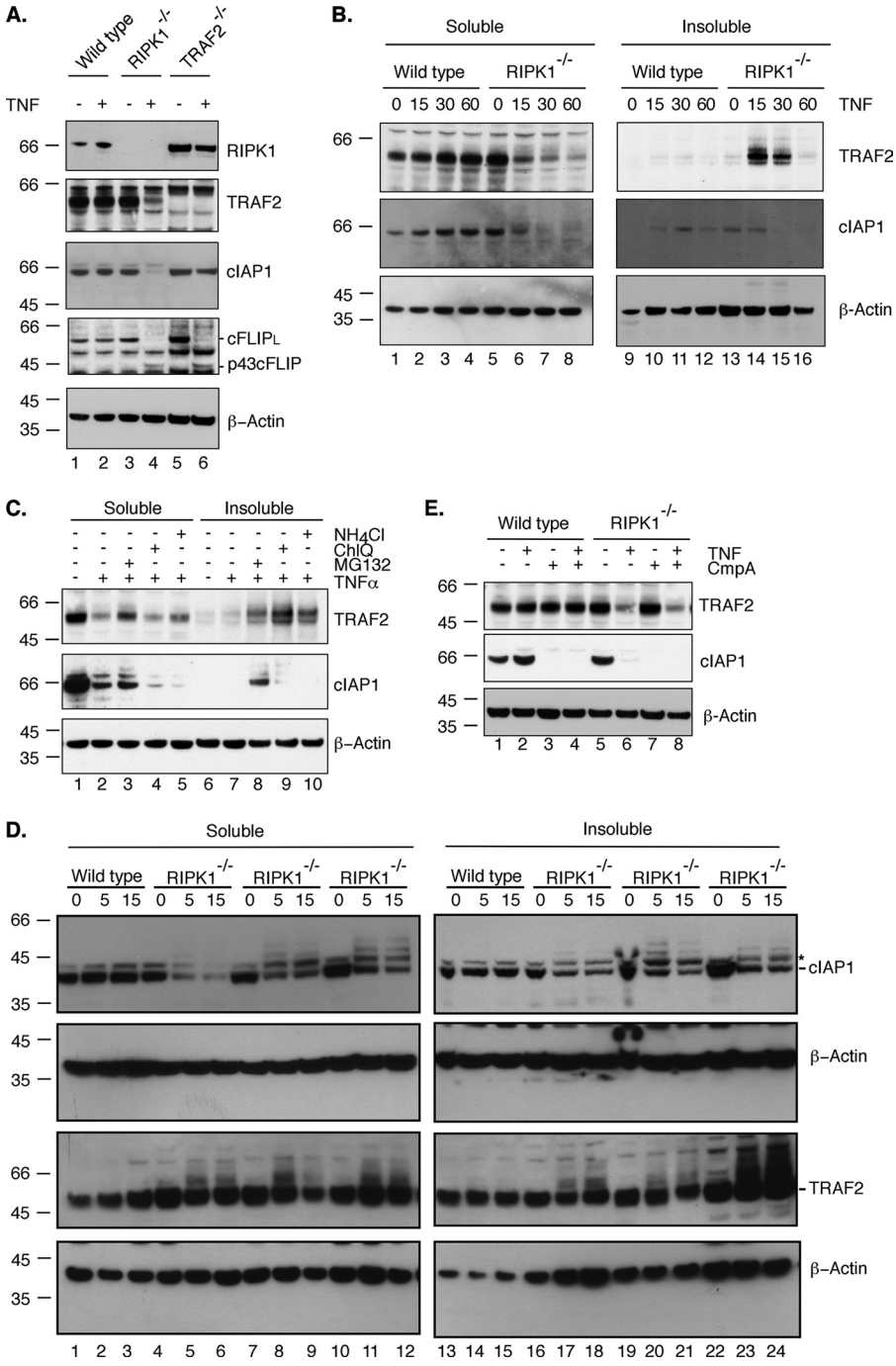

FIGURE 1. **TRAF2 and cIAP1 are degraded in RIPK1$^{-/-}$ MEFs in response to TNF.** *A*, wild-type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with 100 ng/ml Fc-TNF for 1 h and then lysed with DISC buffer. Proteins were analyzed by Western blot for cIAP1, TRAF2, RIPK1, and cFLIP. *B*, wild-type and RIPK1$^{-/-}$ MEFs were treated with 100 ng/ml Fc-TNF for 0, 15, 30, and 60 min. Cells were then lysed in DISC lysis buffer and split into soluble and insoluble fractions. 25 mg of protein from both fractions was run on SDS-PAGE and analyzed for levels of indicated proteins by Western blot. *C*, RIPK1$^{-/-}$ MEFs were treated with either 10 μM MG132, 100 mM NH4Cl, or 200 mM chloroquine for 1 h followed by treatment with 100 ng/ml Fc-TNF for 1 h. DISC lysates were then separated into soluble and insoluble and levels of indicated proteins detected by Western blot. *D*, wild-type and RIPK1$^{-/-}$ MEFs were treated with either 500 nM compound A or untreated for 1 h followed by addition of 100 ng/ml Fc-TNF for 1 h. DISC lysates were made and the soluble and insoluble fraction was analyzed for levels of cIAP1 and TRAF2 by Western blot.

a clear laddering/smearing of both TRAF2 and cIAP1 after TNF treatment. While not conclusive, this is suggestive of ubiquitylation.

The E3 ligases cIAP1 and cIAP2 are able to ubiquitylate both themselves and TRAF2 (19–21). TRAF2 interacts with cIAPs via specific cIAP-interacting motif (3). To determine if cIAPs were responsible for the TNF-induced degradation of TRAF2 and cIAP1, wild-type and RIPK1$^{-/-}$ MEFs were pretreated with the IAP antagonist compound A for 1 h, to degrade cIAPs, before the addition of TNF. Because pretreatment of RIPK1$^{-/-}$ MEFs with compound A had no effect on the loss of TRAF2, cIAPs are not required for TNF-induced TRAF2 degradation (Fig. 1*E*). We were unable to confirm that reconstitution of RIPK1 in the RIPK1$^{-/-}$ MEFs reversed this phenotype as we

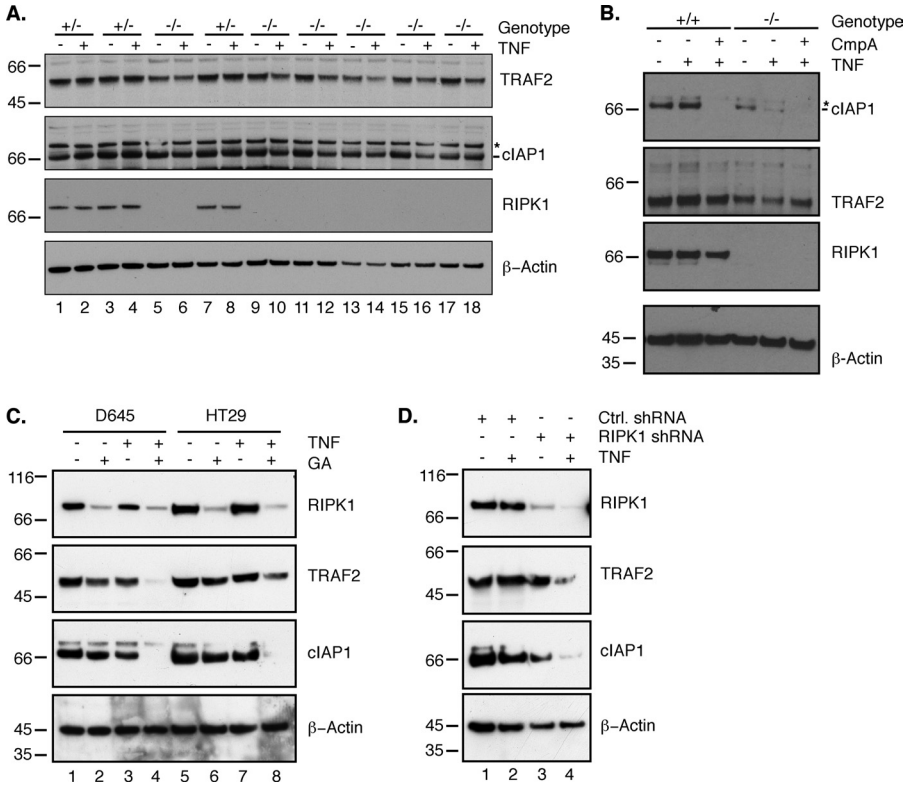

FIGURE 2. **TRAF2 and cIAP1 are degraded in primary tissues treated with TNF.** *A*, E19 embryos were taken and thymus was harvested. Keratinocytes were isolated from day old RIPK$^{-/-}$ 1-day-old pups. Thymi were dispersed into single cell suspensions and half of each was left untreated while the other half was treated with 100 ng/ml Fc-TNF for 1 h. Cells were lysed in DISC lysis buffer and analyzed for TRAF2 and cIAP1 by Western blot. Genotypes were confirmed by Western blot and PCR. *B*, primary keratinocytes were trated with 100 ng/ml Fc-TNF for 1 h or 500 nM compound A as a positive control for cIAP1 degradation, followed by lysis in DISC buffer and proteins detected by Western blot. *C*, D645 and HT29 cells were pretreated with 500 nM geldanamycin for 16 h followed by treatment with 100 ng/ml Fc-TNF for 1 h followed by lysis in DISC buffer and analyzed for protein levels by Western blot. *D*, HT29 cells were infected an lentiviral shRNA against RIPK1 or a control shRNA, and cells were then treated with 100 ng/ml Fc-TNF for 1 h followed by lysis in DISC buffer and analyzed for protein levels by Western blot.

consistently find that exogenous expression of RIPK1 in MEFs causes apoptotic death, even in RIPK1$^{-/-}$ cells (data not shown).

TNF-induced TRAF2 and cIAP1 Degradation Occurs in Multiple RIPK1$^{-/-}$ Cell Types—The tissues that are most affected by deletion of RIPK1 in mice are the thymus and spleen (2). To confirm that degradation of TRAF2 and cIAP1 occurs in primary cell types, thymocytes were taken from E19 embryos from RIPK1± timed matings and treated with TNF for 1 h. TRAF2 and cIAP1 were both degraded in RIPK1$^{-/-}$ thymocytes after TNF treatment (Fig. 2*A*, *lanes 5–6* and *9–18*), while in thymocytes from RIPK1$^{+/-}$ embryos, no degradation was observed. While some variation in the degree of TRAF2 and cIAP1 degradation can be seen, it is clear that there is degradation of either TRAF2 or cIAP1 in all RIPK1$^{-/-}$ thymocytes in response to TNF.

In addition to thymocytes, primary keratinocytes were generated from timed matings of RIPK1$^{+/-}$ mice. The keratinocytes were treated with TNF and the levels of cIAP1 and TRAF2 analyzed by Western blot (Fig. 2*B*). In the wild-type embryos no TRAF2 or cIAP1 degradation was seen. In contrast there was a reduction in the levels of both cIAP1 and TRAF2 in the RIPK1$^{-/-}$ keratinocytes treated with TNF, further confirming this phenotype in multiple primary cell lineages.

To determine whether TNF would also cause loss of TRAF2 in the absence of RIPK1 in human cancer lines D645 (glioma) and HT29 (colon cancer) by either Geldanamycin treatment or RIPK1 shRNA expression. Geldanamycin has been shown to induce RIPK1 degradation (22). With either approach of RIPK1 depletion, there is a clear loss of TRAF2 and cIAP1 after TNF treatment confirming that RIPK1 protects TRAF2 and cIAP1 from TNF-induced degradation in human cells as well (Fig. 2, *C* and *D*).

Signals Leading to Degradation of TRAF2 and cIAP1 Come from TNFR1 and Not TNFR2—TNF can ligate both TNFR1 and TNFR2. TNFR2 signaling has been reported to lead to degradation of TRAF2 and subsequent activation of non-canonical NF-κB (20, 23). TNFR2 has a higher affinity for the membrane-bound form of TNF (24). The Fc-TNF fusion protein that we used is thought to closely mimic the membrane-bound form of TNF (24). Thus we wondered if the degradation of TRAF2 and cIAP1 seen in RIPK1$^{-/-}$ MEFs may be due to Fc-TNF ligand causing activation of TNFR2 receptors.

To determine whether TNFR1 or TNFR2 was the relevant receptor in these experiments, wild-type and RIPK1$^{-/-}$ MEFs were treated with soluble mouse TNF for 1 h. Soluble TNF ligand is unable to trigger TNFR2 mediated TRAF2 degradation (23). Despite this, there was a clear loss of TRAF2 in the RIPK1$^{-/-}$ MEFs, consistent with the notion that TNFR1 signaling caused TRAF2 and cIAP1 degradation (Fig. 3*A*). However, because the degree of degradation appeared to be less than with Fc-TNF, we treated TNFR1$^{-/-}$ RIPK1$^{-/-}$ DKO MEFs

with the Fc-TNF ligand and assessed the degradation of TRAF2 and cIAP1. In the absence of both TNFR1 and RIPK1 there was significantly less degradation of TRAF2 and cIAP1 (Fig. 3*B*), confirming that TNFR1 is the main receptor required for TNF to trigger degradation of TRAF2 and cIAP1 in RIPK1$^{-/-}$ MEFs.

Whereas the role of RIPK1 in TNFR1 signaling has been extensively studied, its role in signaling from other TNF receptor family members is less clear. To determine if the absence of RIPK1 would lead to degradation of TRAF2 after ligation of other TNFSFR members, we looked at both CD95 and TRAIL receptor signaling in RIPK1$^{-/-}$ MEFs, because both CD95 and TRAIL receptors have been reported to recruit RIPK1 and TRAF2, and both have a death domain (25–27). Despite their similar signaling mechanisms, neither ligand-induced TRAF2 degradation in RIPK1$^{-/-}$ MEFs (Fig. 3*C*).

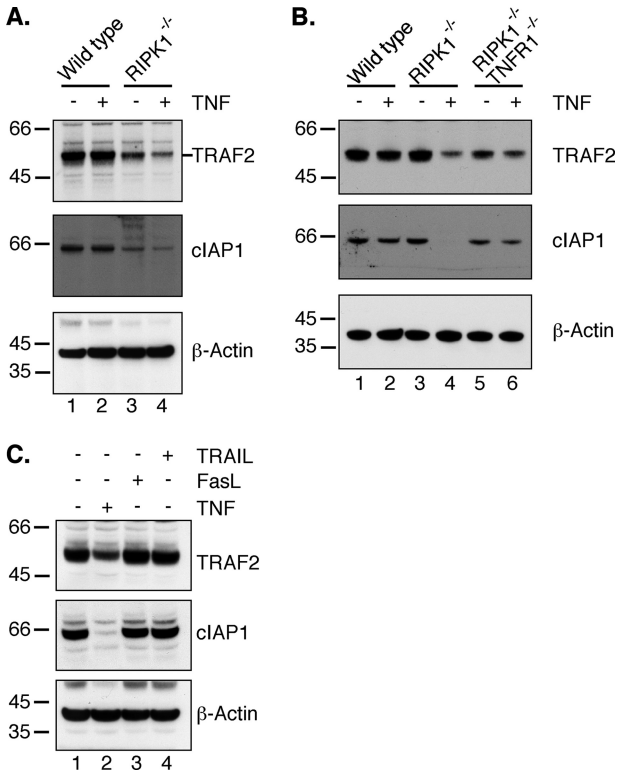

FIGURE 3. **TNFR1 mediates the degradative signal for TRAF2 and cIAP1.** *A*, wild-type and RIPK1$^{-/-}$ MEFs were left untreated or treated with 100 ng/ml soluble mmTNF for 1 h and DISC lysates analyzed for the levels of TRAF2 and cIAP1. *B*, wild-type, RIPK1$^{-/-}$, and RIPK1$^{-/-}$ TNFR1$^{-/-}$ double knock-out MEFs were treated with 100 ng/ml Fc-TNF for 1 h and levels of TRAF2 and cIAP1 were assessed. *C*, wild-type and RIPK1$^{-/-}$ MEFs were treated with either 10 ng/ml of FasL or 1 mg/ml of Iso Leu TRAIL for 1 h, and lysates were analyzed for levels of TRAF2 and cIAP1.

The Kinase Activity of RIPK1 Is Not Required to Prevent TRAF2 and cIAP1 Degradation—To determine if the kinase activity of RIPK1 was necessary to protect TRAF2 and cIAP1 from TNF induced degradation, wild-type and RIPK1$^{-/-}$ MEFs were pretreated with the RIPK1-specific kinase inhibitor necrostatin (28) at concentrations shown to inhibit RIPK1-dependent cell death (Ref. 29 and supplemental Fig. S2). The MEFs were then exposed to TNF or TNF and cycloheximide for 1 h, and levels of TRAF2 and cIAP1 were analyzed by Western blot. Levels of TRAF2 were unaffected by addition of necrostatin, either in the presence or absence of TNF (Fig. 4*A*, *lane 4* and *lanes 5–8*).

Wild-type and RIPK1$^{-/-}$ cells pretreated with necrostatin were also treated with TNF and TNF plus low dose cycloheximide for 24 h, and viability was assessed by propidium iodide (PI) exclusion using flow cytometry. As well as having no effect on TRAF2 degradation, necrostatin did not alter the sensitivity of wild type MEFs to apoptosis triggered by TNF (Fig. 4*B*). The lack of requirement for the kinase activity of RIPK1 suggests that RIPK1 may be acting to protect TRAF2 and cIAP1 via a physical or interaction based mechanism, rather than by phosphorylating a substrate.

TNF Induces Activation of Non-canonical NF-κB in RIPK1$^{-/-}$ MEFs—Although transformed RIPK1$^{-/-}$ B-cells have impaired activation of NF-κB in response to TNF (2), we have recently shown that RIPK1$^{-/-}$ MEFs can activate canonical NF-κB normally in response to TNF (1). On the other hand, loss of TRAF2 or cIAPs leads to activation of non-canonical NF-κB, even in cells that have not been treated with cytokine (12, 13, 21, 31, 32). The loss of TRAF2 and cIAP1 seen in TNF-treated RIPK1$^{-/-}$ MEFs suggested that there might be an

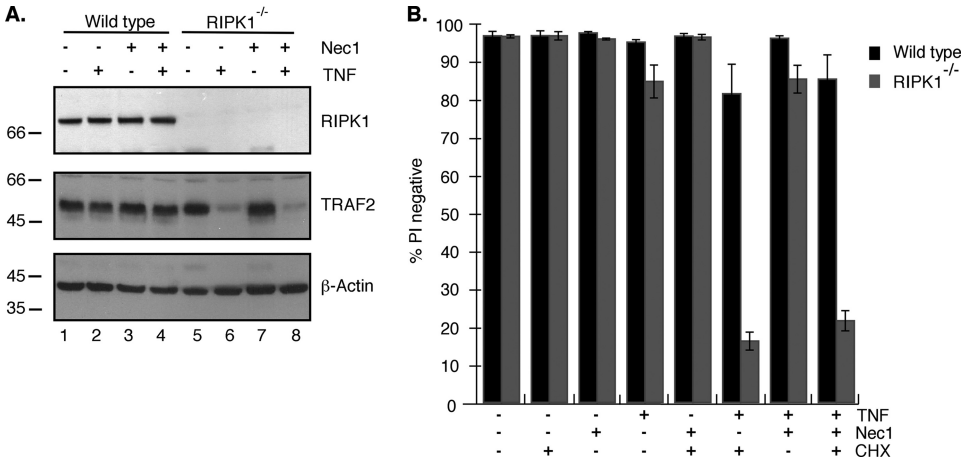

FIGURE 4. **The kinase activity of RIPK1 is not required for protection of TRAF2 and cIAP1.** *A*, wild-type and RIPK1$^{-/-}$ MEFs were pretreated with NEC-1 followed by treatment with 100 ng/ml Fc-TNF for 1 h and lysed in DISC followed by detection of TRAF2 levels by Western blot. *B*, wild-type and RIPK1$^{-/-}$ MEFs were treated with NEC-1 for 1 h prior to treatment with 100 ng/ml Fc-TNF for 24 h. Cells were isolated and stained with propidium iodide and analyzed for uptake by flow cytometry. Error bars show S.E. of at least three independent experiments. To control for NEC-1 activity, L929 cells.

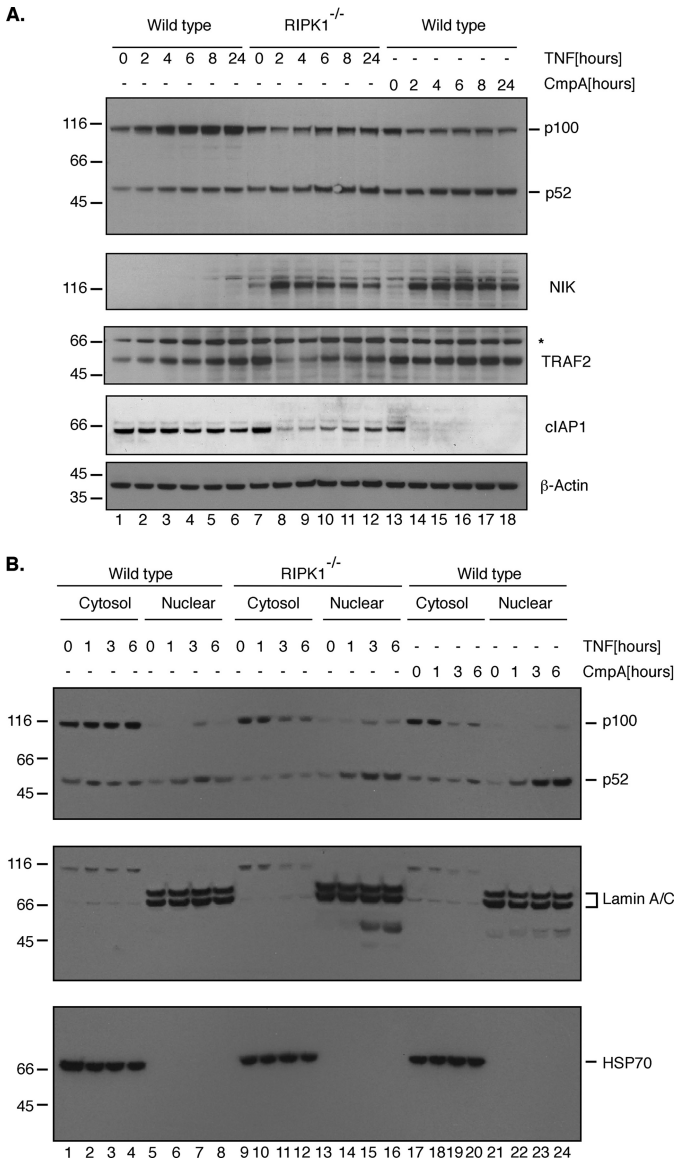

FIGURE 5. **TNF induces non-canonical NF-κB in RIPK1$^{-/-}$ MEFs.** *A*, wild-type and RIPK1$^{-/-}$ MEFs were treated with 100 ng/ml Fc-TNF for the times shown. In parallel, wild-type MEFs were also treated with 500 nM compound A for the same times. Cells were lysed in DISC buffer and analyzed for the levels of NIK, cIAP1, and TRAF2, and processing of p100 to p52. *B*, wild-type and RIPK1$^{-/-}$ MEFs were treated with 100 ng/ml of Fc-TNF for the indicated times followed by fractionation into cytosolic and nuclear fractions. Fractions were then analyzed by Western blot for the levels of p100/p52 and Lamin A/C and Hsp70 as a loading controls.

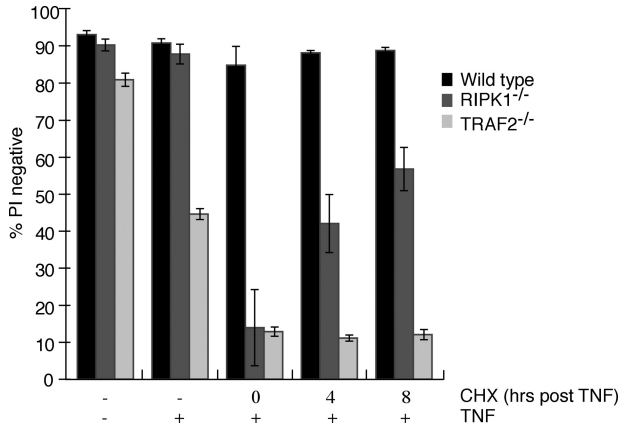

FIGURE 6. **Loss of TRAF2 is responsible for the sensitivity of RIPK1$^{-/-}$ MEFs to TNF.** Wild-type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were left untreated or treated with 100 ng/ml Fc-TNF for 24 h. 250 ng/ml CHX was added immediately, 4 h or 8 h after TNF addition, and cells were analyzed for viability by PI uptake using flow cytometry.

induction of non-canonical NF-κB in addition to the canonical NF-κB response. To test this, both wild-type and RIPK1$^{-/-}$ MEFs were treated with TNF for various times, and lysates were analyzed for markers of non-canonical NF-κB activation, namely degradation of p100 and production of p52. Wild-type MEFs were also treated with compound A to degrade cIAPs as a positive control (31).

Western blot assays showed that steady-state levels of non-canonical NF-κB and NIK were slightly higher in RIPK1$^{-/-}$ MEFs compared with wild-type MEFs (Fig. 5*A*, *lanes 1* and *7*). However, following addition of TNF there was a strong and sustained activation of non-canonical NF-κB with processing of p100 to p52 and stabilization of NF-κB-inducing kinase (NIK) (Fig. 5*A*, *lanes 7–12*). Furthermore, the increase in NIK levels and processing of p100 to p52 in TNF-treated RIPK1$^{-/-}$ MEFs was similar to that caused by depletion of cIAPs by smac-mimetic compound in wild-type MEFs (Fig. 5*A*, *lanes 13–18*, and Ref. 31).

Supporting the idea that the increase in NIK levels is a consequence of TRAF2 and cIAP1 degradation, there was a strong correlation between the loss of TRAF2 and cIAP1 and the subsequent stabilization of NIK and processing of p100 in response to TNF stimulation. Over 24 h, TRAF2 and cIAP1 levels began to recover, and there was a concomitant decrease in the levels of NIK (Fig. 5*A*, *lanes 7–12*).

To confirm that NIK stabilization resulted in induction of non-canonical NF-κB signaling, the nuclear translocation of p52 was assessed. In RIPK1$^{-/-}$ MEFs treated with TNF, the levels of nuclear p52 increase with similar kinetics to those in wild-type cells treated with the IAP antagonist compound A (Fig. 5*B*, *lanes 9–16* and *17–24*). Correspondingly, wild-type cells treated with TNF showed no significant increase in nuclear p52 (Fig. 5*B*, *lanes 1–8*).

TRAF2 Degradation Is Responsible for RIPK1$^{-/-}$ Sensitivity to TNF—If the loss of cIAP1 and TRAF2 were responsible for the sensitization of RIPK1$^{-/-}$ MEFs to killing by TNF plus low doses of CHX, we reasoned that because cIAP1 and TRAF2 levels partially rebounded after several hours of TNF treatment (Fig. 5, *lanes 8–12*), addition of CHX at these later time points might not result in as much death. To test this, wild type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with TNF, and low doses of CHX were added immediately, 4 h or 8 h afterward. Cells were then analyzed for PI uptake by flow cytometry. In RIPK1$^{-/-}$ MEFs, addition of CHX 4 and 8 h after TNFα addition caused less cell death compared with immediate addition of CHX (Fig. 6). In contrast, TRAF2$^{-/-}$ MEFs showed no decrease in sensitivity to TNF when CHX was added at later time points, suggesting that loss of TRAF2 is the key step in sensitizing RIPK1$^{-/-}$ MEFs to TNF.

RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs Show Activation of Caspase-8 following TNF Treatment—Whereas RIPK1$^{-/-}$ MEFs treated with TNF activate NF-κB at the same rate as

wild-type MEFs (1), RIPK1$^{-/-}$ MEFs are nonetheless much more likely to die when treated with TNF plus low dose cycloheximide (Fig. 5*A* and Ref. 1, 2). In this respect RIPK1$^{-/-}$ MEFs resemble TRAF2 knock-out MEFs, which are also much more sensitive to TNF and low dose cycloheximide than to TNF alone (11). TNF-induced apoptosis is mediated by caspase-8. To examine caspase-8 activation in RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs, cells were treated with TNF or TNF plus cycloheximide for 6 h. Lysates were analyzed for cleavage of caspase-8 and PARP, a caspase-3 substrate. In RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs treated with TNF, there was cleavage of caspase-8 to its p43 form (Fig. 7*A*, *lanes 5* & *11*). Addition of CHX in combination with TNF resulted in increased cleavage of PARP and caspase-8 to its p43 form in both genotypes (Fig. 7*A*, *lanes 6* & *12*), and the generation of the p18 form of caspase-8 can be seen in the RIPK1$^{-/-}$ MEFs (Fig. 6*B*, *lane 12*). In both genotypes, cleavage of PARP was seen, suggesting activation of caspase-3. In contrast, in wild-type cells, neither TNF nor TNF plus CHX resulted in significant processing or cleavage or caspase-8 or PARP.

TNF-mediated Transcription of a Pro-survival Protein Protects RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs from Death—It is commonly inferred that CHX sensitizes cells to TNF by preventing translation of pro-survival proteins such as cFLIP, which are otherwise induced by NF-κB signals from TNFR1 (33). The fact that the low doses of CHX that can sensitize both TRAF2$^{-/-}$ and RIPK1$^{-/-}$ cells to killing by TNF do not allow TNF to kill wild-type cells, whereas high doses of CHX do sensitize wild-type cells to TNF, suggests that low doses of CHX do not block translation completely.

To test whether inhibition of transcription could sensitize cells to TNF, just as CHX could by inhibiting translation we treated cells with the transcription inhibitor actinomycin D. If the effect of low doses of CHX were due to a result of inhibition of translation of a transcriptionally induced pro-survival protein, then the same effect should be seen with inhibition of transcription. Indeed, this appears to be the case, because inhibition of transcription with actinomycin D allowed TNF to induce death of RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs with very similar kinetics to those caused by inhibition of translation with CHX (Fig. 7*B*). This supports the idea that there is a transcriptionally up-regulated protein(s) protecting RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs from being killed by TNF alone. Furthermore, these observations would be consistent with a mechanism in which RIPK1 protects the cells by stabilizing TRAF2, and TRAF2 is needed for the signals that up-regulate transcription of a gene for a cell death inhibitory protein.

cFLIP$_L$ Is Destabilized in RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs—A key regulator of caspase-8 activation that is known to be transcriptionally controlled, and may be destabilized by TNF, is cFLIP (3, 33, 34). As seen in Fig. 1*A*, the levels of cFLIP$_L$ decline significantly within 1 h of TNF treatment of RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs, and there is a concomitant appearance of p43cFLIP, the caspase-8 cleavage product of cFLIP$_L$. The rapid loss of cFLIP$_L$ and appearance of a relatively small amount of p43cFLIP in TNF-treated RIPK1$^{-/-}$ and TRAF2$^{-/-}$ cells, suggests that there is both cleavage of cFLIP$_L$ by caspase-8, as well as a signal that targets cFLIP$_L$ for degradation. To examine this,

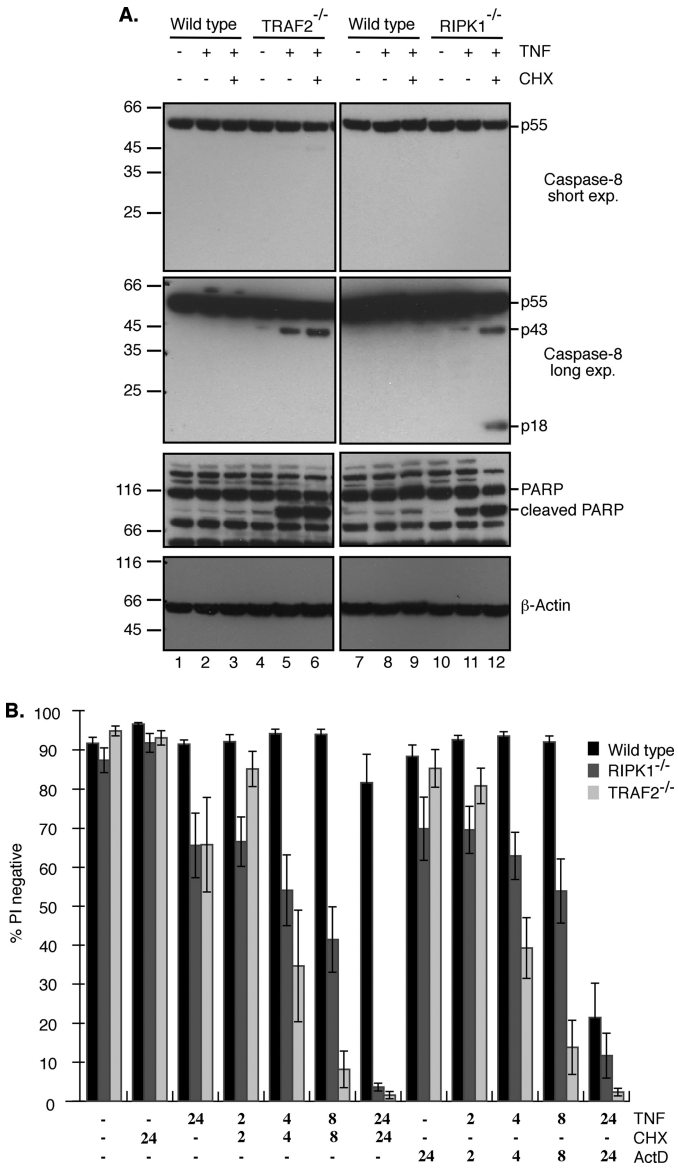

FIGURE 7. **RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs transcriptionally up-regulate a pro-survival protein, and show activation of caspase-8.** *A*, wild-type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with either 100 ng/ml Fc-TNF for 6 h or 100 ng/ml Fc-TNF plus 250 ng/ml of CHX for 6 h. Cells were lysed in DISC buffer and cleavage of caspase-8 and PARP were analyzed by Western blot. *B*, wild-type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with either 250 ng/ml of CHX or 25 ng/ml of actinomycin D in combination with 100 ng/ml Fc-TNF for 2 or 4 h. As a control, cells were also treated for 24 h with 100 ng/ml Fc-TNF, 250 ng/ml of CHX, and 25 ng/ml of actinomycin D alone. Cells were harvested and stained with PI followed by analysis by flow cytometry. Error bars show S.E. of at least three independent experiments.

wild type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with the proteasomal inhibitor MG132 for 1 h prior to treatment with TNF. Cells were lysed in DISC lysis buffer and probed for cFLIP. MG132 pretreatment prevented loss of cFLIP$_L$ in both RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs, but had only a minor effect on the levels of p43cFLIP, even reducing it to some extent (Fig. 8*A*, *lanes 6* and *9*). This indicates that, in the absence of RIPK1$^{-/-}$ or TRAF2$^{-/-}$, either TNF causes activation of an MG132-inhibitable process that degrades cFLIP$_L$, or that cFLIP$_L$ is constantly being degraded by an MG132-inhibitable process, but RIPK1 and TRAF2 are needed to replenish it.

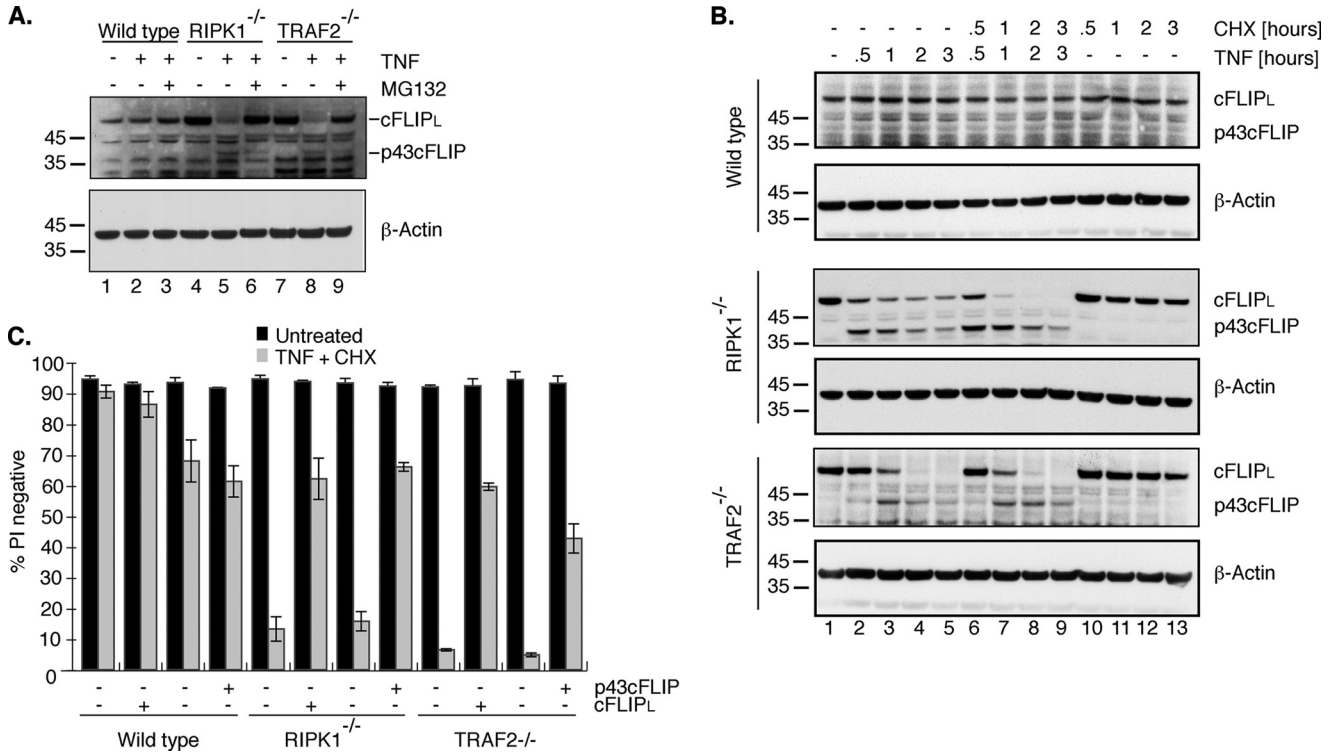

FIGURE 8. **cFLIP$_L$ is destabilized in RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs.** *A*, wild-type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated either with MG132 for 1 h prior to treatment with 100 ng/ml Fc-TNF for 1 h, or 100 ng/ml Fc-TNF for 1 h alone. Cells were lysed in DISC buffer and levels of cFLIP$_L$ and p43cFLIP were detected by Western blot. *B*, wild-type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with either 100 ng/ml Fc-TNF, 250 ng/ml CHX, or a combination of both for the indicated times. Levels of cFLIP and cLFIP-43 were detected by Western blot. *C*, wild-type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were infected with 4HT inducible lentiviral constructs coding for cFLIP$_L$ or p43cFLIP. Constructs were either induced or not overnight and then treated with 100 ng/ml Fc-TNF for 24 h. Cells were harvested and stained with PI and analyzed for uptake by flow cytometry. Error bars represent S.E. from at least three independent experiments.

RIPK1$^{-/-}$ MEFs are slightly more sensitive than wild-type MEFs to treatment with TNF alone while TRAF2$^{-/-}$ MEFs exhibit somewhat higher sensitivity (Fig. 6) (1, 11). The rapid cleavage of cFLIP$_L$ to p43cFLIP, and equally rapid destabilization of cFLIP$_L$, suggests that p43cFLIP may be sufficient to prevent enough activation of caspase-8 to cause cell death. Inhibition of transcription or translation, even slightly, may be sufficient to tip the scales in favor of cell death by reducing the levels of p43cFLIP, thereby allowing further cleavage and activation of caspase-8. To examine the half-life of cFLIP$_L$ and p43cFLIP in the presence of low dose cycloheximide, wild type, RIPK1$^{-/-}$, and TRAF2$^{-/-}$ MEFs were treated with CHX, TNF, or both for various times, and levels of cFLIP were assessed by Western blot. As can be seen in Fig. 8*B*, cFLIP$_L$ was cleaved to p43cFLIP and also degraded more rapidly in RIPK1$^{-/-}$ and TRAF2$^{-/-}$ MEFs treated with TNF or TNF plus CHX (Fig. 8*B*). p43cFLIP remained present over the 3-h time course showing that it either has a longer half-life, or that any newly translated cFLIP$_L$ was being rapidly cleaved and degraded at a rate that maintained p43cFLIP levels. In comparison, wild-type cells showed no cFLIP$_L$ cleavage when treated with TNF alone, or TNF plus low dose CHX. Addition of CHX reduced the level of p43cFLIP and cFLIP$_L$ over time, coinciding with the onset of death and the appearance of cleaved caspase-8 at around 4 h after addition of TNF plus CHX (Figs. 8*B* and 7*B*).

Overexpression of cFLIP$_L$ can protect TRAF2$^{-/-}$ MEFs from killing by TNF (35). If loss of cFLIP$_L$ is the cause of death in RIPK1$^{-/-}$ MEFs, exogenous expression of cFLIP$_L$ or p43cFLIP should be able to prevent caspase-8 activation and death. To confirm this in both TRAF2$^{-/-}$ MEFs and RIPK1$^{-/-}$ MEFs, cells were infected with inducible lentiviral constructs expressing either cFLIP$_L$ or p43cFLIP. Cells were then left untreated or treated with TNF plus low dose CHX and assayed for cell death. Induction of either form of cFLIP was able to protect most of the cells of both genotypes from death caused by TNF plus low dose CHX. (Fig. 8*C*). This indicates that the mechanism of death triggered by TNF is capable of being inhibited by both cFLIP$_L$ and p43cFLIP.

DISCUSSION

Although RIPK1 can promote cell death in certain circumstances, it is clear that RIPK1 can also promote survival of TNF-treated cells. For many years it was thought that this was due to a requirement for RIPK1 to activate canonical NF-κB and induce pro-survival genes such as cFLIP and cIAPs. While RIPK1 ubiquitylation and recruitment of IKKs clearly plays a part in TNF induced NF-κB activation in some cell types, the finding that RIPK1 is not essential for TNF to activate canonical NF-κB in MEFs suggested some other function of RIPK1 is responsible for its pro-survival effects (1). Our finding that in the absence of RIPK1, addition of TNF caused rapid degradation of TRAF2, cIAP1, and cFLIP$_L$ now provides an additional mechanism to explain why RIPK1$^{-/-}$ cells are highly sensitive to TNF plus low doses of cycloheximide, and why they share this phenotype with TRAF2$^{-/-}$ cells.

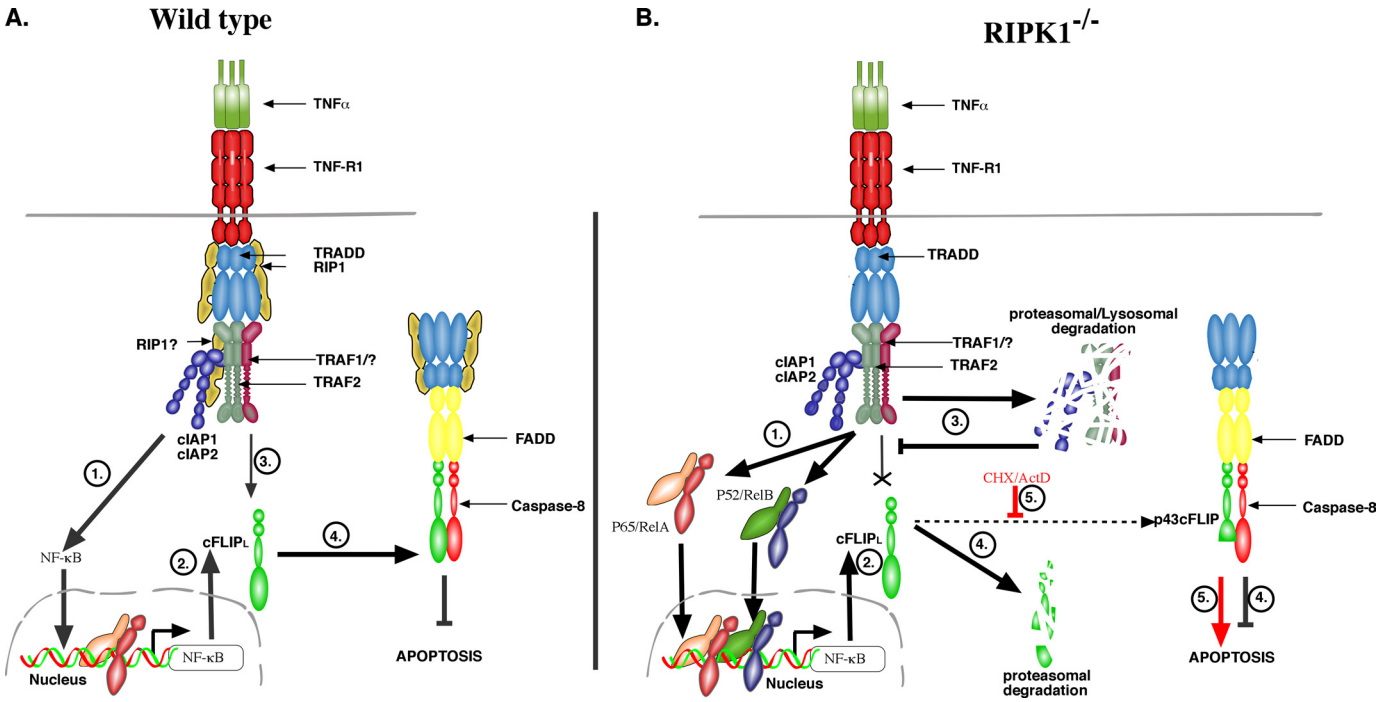

FIGURE 9. **Model of pro-death and pro-survival functions of RIPK1.** *A*, wild-type cells. *Step 1*, TNF binding triggers assembly of complex I by recruitment of TRAFs and cIAPs, which results in p65/RelA NF-κB translocation to the nucleus. *Step 2*, $cFLIP_L$ is up-regulated by NF-κB. *Step 3*, in the presence of RIPK1, TRAF2, and cIAP1 are stabilized (potentially by direct interaction with RIPK1, but it does not require RIPK1 kinase activity) and TRAF2 mediates stabilization of $cFLIP_L$ by an unknown mechanism. *Step 4*, $cFLIP_L$ binds to caspase-8, blocking its activation and preventing apoptosis. *B*, pro-survival effect of RIPK1. *Step 1*, TNF binding triggers assembly of complex I by recruitment of TRAFs and cIAPs which results in NF-κB translocation to the nucleus. *Step 2*, $cFLIP_L$ is up-regulated by NF-κB. *Step 3*, in the absence of RIPK1, cIAP1 and TRAF2 are degraded by a proteasomal/lysosomal mechanism. This also leads to NIK stabilization and p100 processing (not shown), and translocation of p52/RelB dimers to the nucleus. *Step 4*, loss of TRAF2 leads to destabilization of $cFLIP_L$, the majority of which is degraded by a proteasomal mechanism. A proportion of $cFLIP_L$ interacts with caspase-8 and is cleaved to p43cFLIP, thereby blocking full caspase-8 activation. *Step 5*, partial inhibition of transcription or translation reduces levels of $cFLIP_L$ and p43cFLIP allowing caspase-8 activation, resulting in apoptosis.

The fact that cIAP1 was rapidly degraded after TNF was added to the $RIPK1^{-/-}$ MEFs raised the possibility that death of the cells was due to the absence of cIAP1 (21, 31, 36, 37). However, we do not favor this notion, because previous experiments with cIAP1 gene-deleted cells, or cells treated with an IAP antagonist that depletes cells of cIAP1, showed that killing by TNF could be blocked by the RIPK1 kinase inhibitor necrostatin or genetic deletion of RIPK1 (1, 37). These experiments are consistent with observations from Wang *et al.* (37) that TNF can induce two differentially regulated caspase 8-dependent pathways. In addition, there is no observable loss of cIAP1 in $TRAF2^{-/-}$ MEFs treated with TNF (Fig. 1*A*), yet $TRAF2^{-/-}$ MEFs exhibit the same phenotype as $RIPK1^{-/-}$ MEFs in response to TNF.

Our results are consistent with a model in which TNF activates canonical NF-κB in wild-type MEFs, but does not induce cell death, because RIPK1 is recruited to a complex associated with TNFR1, where it becomes ubiquitylated, allowing recruitment of further proteins. In this way, RIPK1 plays a non-essential role in activation of p65/RelA NF-κB, but also somehow protects TRAF2, cIAP1 and cFLIP from degradation (Fig. 9*A*). These proteins prevent activation of caspase-8. In the presence of TNF plus low dose CHX, wild type MEFs still survive, but those derived from $RIPK1^{-/-}$ or $TRAF2^{-/-}$ mice do not. We believe this is because when TRAF2 is absent, $cFLIP_L$ is destabilized and activation of caspase 8 is no longer blocked (Fig. 9*B*). Addition of CHX is required for TNF to cause death of $RIPK1^{-/-}$ or $TRAF2^{-/-}$ MEFs due to the level of transcriptionally up-regulated production of $cFLIP_L$ and the rate of its degradation and cleavage being in equilibrium. When translation or transcription is inhibited, even at levels that have no adverse effects on wild-type cells, then the amount of $cFLIP_L$ and p43cFLIP are reduced enough to allow further processing of caspase 8 and death in response to TNF. Consistent with this model, we were able to detect minimal levels of $cFLIP_L$ in $RIPK1^{-/-}$ MEFs treated with TNF alone, but $cFLIP_L$ was rapidly reduced to undetectable levels in cells treated with TNF plus low dose CHX (Fig. 9*B*).

Observations suggesting that the RING domain of TRAF2 is important for its pro-survival function may provide clues to the mechanism of TRAF2s cytoprotective activity (1, 3, 9). Whereas we do not yet know how RIPK1 prevents destabilization of TRAF2, experiments with the kinase inhibitor necrostatin show that RIPK1 kinase activity is not required for its pro-survival activity. This is in marked contrast to RIPK1 pro-death activity when caspases are blocked or IAP antagonists used in combination with TNF, which can be blocked with necrostatin or kinase domain mutation and hence does require its kinase activity (27, 28, 37–39). One possibility is that in complex I, RIPK1 prevents a ubiquitin E3 ligase from access to TRAF2 and cIAP1.

These results explain why $RIPK1^{-/-}$ and $TRAF2^{-/-}$ MEFs respond in a similar way to TNF plus low dose CHX, and may also explain similarities in the phenotypes of $RIPK1^{-/-}$ and $TRAF2^{-/-}$ mice. Mice of either genotype exhibit early postnatal lethality with severe reduction in thymocyte and splenocyte

populations (2, 40). Several studies have shown that crossing TRAF2$^{-/-}$ and TRAF3$^{-/-}$ mice, which both exhibit constitutive non-canonical NF-κB, with IKKγ/Nemo mutant *Aly* mice or NF-κB2$^{-/-}$ mice protected them from death and rescued their lymphocyte populations (12, 13, 30). Likewise, degradation of TRAF2 and cIAP1 in RIPK1$^{-/-}$ mice may be causing an NF-κB2-dependent death of lymphocytes.

These new findings characterizing the pro-survival activity of RIPK1, highlight that far from simply being a scaffold protein in TNFR signaling, RIPK1 seems to be playing a key role in regulating receptor signaling in cooperation with TRAFs and cIAPs. Being able to separate the pro-survival functions of RIPK1 from its pro-death functions will surely help in understanding the exact role RIPK1 is playing in regulation of TNF receptor signaling.

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